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FINAL REPORT

DO RETROVIRUSES PLAY A ROLE IN POST WEANING MULTISYSTEMIC WASTING SYNDROME IN PIGS

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"Do Retroviruses Play a Role in Post Weaning Multisystemic Wasting Syndrome in Pigs"

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Abstract / Summary

Lentiviruses are etiologic agents in wasting syndromes in a variety of species, from cats to human beings. To date lenitiviruses have not been reported in pigs. Based on the clinical signs and lesions, a lentivirus may be involved in the newly described porcine postweaning multisystemic wasting syndrome (PMWS). In order to control PMWS, identification of all involved pathogens should be accomplished. In order to investigate the role of retroviruses in PMWS sensitive enzymatic and molecular diagnostic techniques were employed. Serum and white blood cells were screened for reverse transcriptase activity, an enzyme specific for retroviruses. Tissues were examined for the presence of conserved lentiviral sequences using polymerase chain reaction (PCR). Serum and stimulated leukocytes from the blood and lymph nodes from pigs with PMWS, as well as from control pigs had reverse transcriptase (RT) activity that was detected by the sensitive amp-RT assay . A 257 bp fragment was amplified from DNA from the blood and bone marrow of pigs with PMWS. This fragment was identical in size to conserved lentiviral sequences that were amplified from plasmids containing DNA from several lentiviruses. Cloning and sequencing of the fragment from affected pigs, however, did not reveal homology with the recognized lentiviruses. Together the results of these analyses suggest that the RT activity present in tissues from control and affected pigs is the result of endogenous retrovirus expression, and that a lentivirus is not a primary pathogen in PMWS.

Executive Summary

Postweaning multisystemic wasting syndrome (PMWS), is a new disease that most often affects 5-12 week old pigs, and has been recognized in swine herds in Canada, the United States, and Europe. Clinically, the disease is characterized by progressive weight loss, tachypnea, dyspnea, and jaundice. Lesions in affected pigs include lymphadenopathy, lymphocytic to granulomatous interstitial pneumonia, lymphocytic to granulomatous hepatitis and nephritis. Previously we have demonstrated a strong association between the lesions of naturally occurring PMWS and the presence of a new porcine circovirus. Recently we have reproduced the lesions of PMWS in germ-free pigs using chloroform-extracted filtrates of tissue homogenates and cell cultures derived from affected pigs.

Although we have ruled out the primacy of many other known porcine pathogens in the condition, and reproduced the lesions of PMWS with inocula that are unlikely to contain infectious enveloped viruses, such as retroviruses, many of the clinical and pathologic features of PMWS, including wasting and lymphoproliferative lesions are reminiscent of those caused by lentiviruses such as human immunodeficiency virus (HIV), ovine lentivirus (OvLV), caprine encephalitis virus (CAEV), jembrana disease virus (JDV), and feline immunodeficiency virus (FIV). Moreover, since it is possible that several pathogens may act in a synergistic fashion in the genesis of PMWS, a search for retroviral involvement in the condition was justified.

Endogenous porcine retroviruses have been recognized for more than 20 years. Currently there is renewed interest in porcine retroviruses, due primarily to the use of pig tissues in xenotransplantation in human beings. Recent research has focused on identifying factors that control porcine retroviral expression and examining the potential of these agents as pathogens in transplant recipients.

Available data indicate that there are multiple copies of endogenous retroviruses in the DNA of domestic pigs, and a new full length endogenous retroviral sequence has recently been described. These studies have used miniature and Yucatan pigs, and there is currently little known about endogenous retroviruses in conventional crossbred pigs. To our knowledge, there has been no evidence to date of a porcine lentivirus.

The objectives of this study were twofold. Firstly to screen for retroviruses in pigs with PMWS using a sensitive generic assay to detect reverse transcriptase activity, and, secondly, to examine tissue from affected pigs for lentiviral pronucleotide sequences. Since lentiviruses have yet to be described in pigs, we employed PCR techniques with degenerate primers which amplify conserved nucleotide sequences from a wide variety of lentivirus, assuming a new lentivirus would share some homology with them.

In order to investigate the role of retroviruses in PMWS, serum and leukocytes were screened for reverse transcriptase activity and tissues were examined for the presence of conserved lentiviral sequences using degenerate primers in a polymerase chain reaction (PCR) reaction. Serum and stimulated leukocytes from the blood and lymph nodes from pigs with PMWS, as well as from control pigs had reverse transcriptase (RT) activity that was detected by the sensitive amp-RT assay . A 257 bp fragment was amplified from DNA from the blood and bone marrow of pigs with PMWS. This fragment was identical in size to conserved lentiviral sequences that were amplified from plasmids containing DNA from several lentiviruses. Cloning and sequencing of the fragment from affected pigs, however, did not reveal homology with the recognized lentiviruses. Together the results of these analyses suggest that the RT activity present in tissues from control and affected pigs is the result of endogenous retrovirus expression, and that a lentivirus is not a primary pathogen in PMWS.

Technical Report

I. SOURCE OF PORCINE TISSUES AND CELL LINES

Tissues, including, blood, liver, lung, kidney, lymphoid nodes and bone marrow were collected from debilitated 5-12 week old pigs exhibiting clinical signs and multisystemic lesions that are characteristic of PMWS. Identical tissues were collected from similarly aged, clinically normal control pigs from a farm which has not had a case of PMWS. In addition, porcine circovirus-tested porcine kidney (PK-15) cells and Madin-Darby bovine kidney (MDBK) cell lines were also used as negative controls.

II. ISOLATION AND ACTIVATION OF CELLS FROM BLOOD, LYMPH NODES AND LUNGS

Blood was collected from the anterior vena cava into acid-citrate-dextrose containing vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of blood / citrate buffer (50% v/v) mixtures layered on Ficol Hypaque (1.077 sp. gravity), washed twice in phosphate buffered saline, and resuspended at 5 x 10^6 cells/ ml in RPMI medium supplemented with HEPES, 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 IU/mL) / streptomycin (100 µg/mL) (supplemented RPMI). Bronchial lymph nodes were aseptically removed and placed in cold RPMI medium in petri dishes on ice. The lymph nodes were teased apart, larger fragments were allowed to settle for 5 min, and the cell-rich supernatant was collected. Lymph node cells were resuspended at 5 x 10^6 / ml in the same supplemented medium used for PBMC. A total of 10^7 PBMC or lymph node cells were cultured in 2 ml of supplemented RPMI in wells of 24 well plates and were stimulated with 2.5 mg/ml concanavalin -A. Pulmonary alveolar macrophages (PAM) were obtained as previously described by bronchial lavage of lungs collected immediately after euthanasia of animals using halothane

inhalation . PAM were resuspended in the supplemented RPMI medium at 10^6 / ml. PAM were not treated with mitogen. Bone marrow was aspirated from the femur via the trochanteric fossa using a Jamshidi needle. Approximately 1 ml of marrow aspirate was suspended in 15 ml of supplemented DMEM with 5% FBS, 1 mg/ml bovine serum albumin, 80 µgm/ml transferrin, and 10^{-5} M hydrocortisone.

III. DETECTION OF REVERSE TRANSCRIPTASE (RT) ACTIVITY BY AMP-RT ASSAY

The Amp-RT assay was performed as previously described. Briefly, this assay measures the ability of a sample to produce a DNA copy of a known heterologous RNA template, and the reverse transcribed cDNA is detected by PCR amplification and Southern blot hybridization. This assay has previously been shown to detect enzyme activity from a wide variety of retroviruses representing all three subfamilies of retroviruses from several host species, including ungulates, and is approximately 100,000 x more sensitive that a standard RT assay.

IV. DNA AND RNA EXTRACTIONS

DNA and RNA used in PCR reactions to detect lentiviral sequences were extracted from tissue samples and cell lines using Tri Reagent according to recommendations of the manufacturer.

V. POLYMERASE CHAIN REACTIONS (PCR), RT-PCR, CLONING AND SEQUENCING

Two sets of primers, termed LV1: 5'-ccggatccdcayccngsaggaytamaa-3' (d: a+t+g; y: c+t; m: a+c), LV2: 5'-ggtctagayryarttcataacccakcca-3' (r: a+g; k: t+g), LV3: 5'-ccggatccgayrtrggkgaygcmta-3' and DDMY: 5'-ccggatccrtcrtccatrta-3' were used in nested PCR reactions as described previously. The design of the primer sequences were based on conserved reverse transcriptase regions of the

polymerase gene of lentiviruses. These primers do not amplify sequences from other known exogenous retroviruses (oncoviruses), nor endogenous retroviruses. The experimental conditions for nested PCR amplifications using the Taq polymerase were identical to those described. The integrity of the extracted DNA in the PCR reactions was assessed by amplification of a sequence from the beta globin gene. Each reaction was performed in a total volume of 25 µl containing 0.1 to 1 µg of DNA. The PCR products were separated in 2% agarose gels and visualized under UV light after staining with ethidium bromide. A slice of agarose gel was cut in the area corresponding to a size of approximately 450 bp (expected size of the amplification product obtained by the first set of primers, LV1-LV2) and DNA was eluted using a Genclean kit. Subsequently, different amounts of eluted DNA were used to amplify a 257 bp fragment using primers LV3-DDMY. In some experiments, the DNA samples from healthy and affected animals were pooled and used for PCR amplification.

Four different recombinant plasmids containing sequences for the following lentiviruses were used as positive control in PCR reactions: feline immundeficiency virus (pFIV-14); caprine arthritis-encephalitis virus (pc63-7e5), equine infectious anemia virus (p29) and human immundeficiency virus (pILIC)

Two micrograms of extracted RNA and 500 ng of reverse primer (LV2) were used for the synthesis of cDNA in a final volume reaction of 20 ml using standard experimental conditions. Subsequently, the synthesized cDNA was used as a source for PCR amplifications as described above. The 257 bp PCR products eluted from agarose gels were cloned into a pCRII vector according to recommendations of the manufacturer. Plasmid DNA from positive recombinants was extracted using a previously described procedure and the LV3-DDMY primers were used to confirm the presence of an insert. Subsequently, nucleotide sequencing was performed using an automated sequencing system.

VI. DETECTION OF RT ACTIVITY.

Reverse transcriptase activity was detected in the serum of randomly chosen pigs with typical signs and lesions of PMWS. As well, RT activity was also detected in the serum of clinically normal age-matched pigs from herds that had diagnosed cases of PMWS, as well as from a closed herd that has never had a known case of PMWS (Table I). Reverse transcriptase activity was also detected in the supernatants of mitogen-stimulated blood mononuclear leukocytes and lymph node cells from a clinically normal pig and pigs with PMWS. Enzymatic activity was not detected in PAM cultures from 4 pigs with PMWS, 3 of which had moderate to severe interstitial pneumonia (Table II).

VII. PCR AMPLIFICATION REACTIONS

To test the ability of the degenerate primers to amplify lentivirus sequences, PCR reactions were performed on four recombinant positive plasmids. The recombinant plasmids, pFIV-14, pc63-7e5, p29 and pILIC used in nested PCR reactions show, as expected, products of 450 bp and 257 bp in length with LV1-LV2 and LV3- DDMY primers respectively (Figure 1). Subsequently, nested PCR reactions were performed on DNA extracted from liver, spleen, lymph node, lung, blood and bone marrow from normal and affected animals. A major amplification product of 257 bp was detected in DNA extracted from two separate pools of blood leukocytes from 15 and 5 affected animals from 2 different herds, from the bone marrow of a single affected animal, and in the positive control pILIC. The same amplification product, although barely detectable, was obtained from DNA extracted from a pool of leukocytes comprising five normal animals, and in normal bone marrow sample from a single normal animal, all from a herd that has not had a case of PMWS (Figure 2). No amplification product was obtained in DNA extracted from liver, lung, lymphoid tissues, spleen and kidney tissues from affected or normal animals, nor

from DNA extracted from PK 15 and MDBK cell lines that were used as negative controls (data not shown). Changes in some reaction parameters, such as the quantity of DNA and annealing temperature, did not alter these results (data not shown). To rule out that the absence of amplification observed in certain tissues was not due the procedure used to extract DNA, a quality control PCR reaction using primers that specifically amplify a region of the beta globin gene was also performed. The presence of a band of the appropriate size was observed in all the samples (data not shown). To determine if a putative porcine lentivirus was actively being expressed in the various tissues of affected animals, RNA samples were also tested by RT-PCR amplification. No amplification product of the appropriate size was observed in any samples tested, including blood and bone marrow (data not shown).

VIII. CHARACTERIZATION OF PUTATIVE LENTIVIRUS-LIKE SEQUENCES BY SEQUENCE ANALYSIS

Since DNA extracted from affected animals produced a more intense band of the appropriate size than normal tissues, it was hypothesized that along with common cellular sequences, an infectious lentivirus agent could have been amplified in the same reaction. To test this, the 257 bp DNA fragment from blood and bone marrow of affected animals was inserted by T tail cloning into the pCRII vector. Recombinant clones identified by PCR reactions using the LV3-DDMY set of primers were subsequently sequenced. Comparison of the nucleotide sequence with existing DNA databases did not reveal homologies with lentivirus-like sequences.

IX. DISCUSSION OF FINDINGS

Our working hypothesis was that a lentivirus is involved in the pathogenesis of PMWS. To test this hypothesis we first applied a highly sensitive assay of RT activity as a generic test for the presence of retroviruses. The

detection of RT activity in serum and supernatants of leukocyte cultures derived from pigs with PMWS was compatible with retroviral infection; however, detection of similar levels of activity in similar samples from clinically normal control pigs suggested that this characteristic enzyme activity was attributable to endogenous retroviral expression, rather than an exogenous retrovirus. Nevertheless, we analyzed DNA extracted from a variety of tissues from affected and normal post weaning pigs using PCR amplification as another sensitive means to determine if this syndrome could be associated with the presence of lentivirus-like sequences. Again, our results did not support the hypothesis.

The primer sequences and conditions used in the PCR reactions were identical to those previously described. These primers were successfully employed to demonstrate the presence of conserved lentiviral polymerase gene sequences from a wide variety of host species in infected cell lines and cell lines containing proviral DNA by use of sets of degenerate primers in a nested PCR reaction. We detected the amplification of a product of approximately 250 bp in blood and bone marrow from affected animals, suggesting that lentivirus-like sequences might be associated with PMWS in pigs. However, the sequencing data obtained through the analysis of recombinant clones containing the 257 bp fragment failed to reveal homology with lentivirus sequences. Similar amplification of irrelevant sequences was previously reported using these PCR.

There are several explanations for these negative results. The first, and the most obvious explanation is that lentiviruses do not play a role in the pathogenesis of PMWS. The production of lesions typical of PMWS with chloroform extracted filtrates containing porcine circo and parvo viruses support this possibility. Secondly, it is possible that only a few cells containing lentiviral particles or lentiviral provirus were present in the various tissues from affected animals that were examined, and this low level of infection was beyond the limits

of detection by PCR amplification. This would be unlike the situation in other species with lentiviral infections, where viremia and lentiviral expression occurs coincident with clinical disease. However, in this context, it is worth noting that previous investigators used, as templates in PCR reactions, DNA extracted from established cell lines containing lentivirus proviral DNA or lentivirus genes as well as highly infected cell lines. Even with those conditions, they found that these primers did not amplify all lentivirus sequences with the same efficiency and in cases like EIAV and bovine immunodeficiency virus, only 20 % of the clones obtained contained lentivirus sequences. Thus, to increase the level of sensitivity in detecting expressed lentivirus sequences, we used RNA templates extracted from different tissues in RT-PCR amplification reactions. However, this also failed to reveal the presence of lentivirus-like sequences in tissues of pigs with PMWS. Finally, we used primers that amplify a region of the reverse transcriptase gene that is highly conserved among lentiviruses isolated from different species, assuming there would be similar homology in a putative porcine lentivirus. It is possible, although we believe unlikely, based on lentiviruses described in other species, that the genome of porcine lentivirus is significantly different from that of other known lentiviruses.

In conclusion, we detected inducible RT activity in primary cell cultures derived from crossbred domestic pigs. These data confirm and extend those recently reported in miniature pigs, and indicate an apparently similar level of infection with endogenous retroviruses in genetically diverse domestic pigs. The fact that RT activity was detected in serum, and at the initiation of PBMC and lymph node cell cultures from some pigs affected with PMWS suggests that the etiologic agent or agents, or the host response to infection, may effect the expression of endogenous retrovirus in vivo. Whether or not such expression of endogenous retrovirus plays a role in the pathogenesis of PMWS is unresolved.

Finally, although we cannot completely rule out the possibility that a new porcine lentivirus is involved in the pathogenesis of PMWS, our failure to detect conserved lentiviral sequences in representative affected pigs with typical clinical signs and lesions of PMWS using these highly sensitive techniques, suggests a lentivirus is not a prevalent or primary pathogen in this emerging infectious disease in swine.

Table I. Detection of reverse transcriptase (RT) activity in the serum of affected with postweaning multisystemic wasting syndrome (PMWS) and clinically normal pigs

pig #	RT activity	source	clinical status
750-1	+	effected herd 1	PMWS
750-2	+	effected herd 1	PMWS
750-3	+	effected herd 1	PMWS
42	-	effected herd 2	normal
41	+	effected herd 2	normal
B43	+	effected herd 2	normal
B44	+	effected herd 2	normal
PSWC-1	-	uneffected herd	normal

Table II. Detection of reverse transcriptase activity in primary cell culture

	days	source of cells				
cells	in culture	PMWS[a] 1	PMWS 2	PMWS 3	PMWS 4	control
PBMC[b]	1	ND	ND	ND	-	+
PBMC	3	+	+	+	-	-
PBMC	7	+	+	+	-	+
BLN[c]	1	-	+	-	ND	-
BLN	3	-	+	+	ND	+
BLN	7	+	+	ND	ND	+
PAM[d]	1	-	-	-	-	ND
PAM	3	-	-	-	-	ND
PAM	7	-	-	-	-	ND

[a]pig affected with postweaning multisystemic wasting syndrome
[b]peripheral blood mononuclear leukocytes
[c]bronchial lymph node cells
[d]pulmonary alveolar macrophages

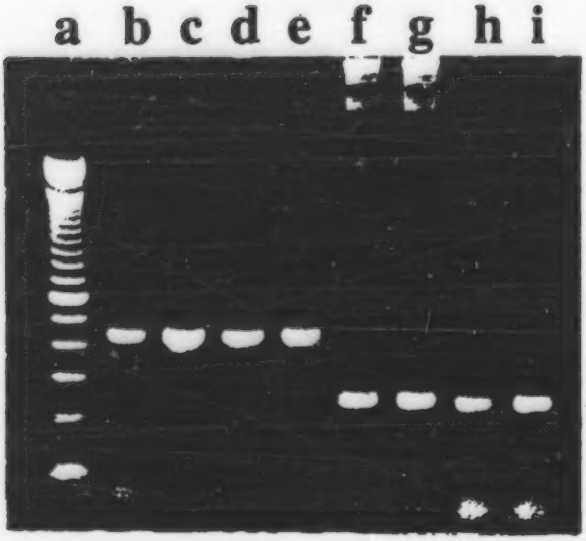

Figure 1. Amplification of different lentiviruses by the LV1-LV2 and LV3-DDMY sets of primers. Ten microliters of reaction products were run in a 2% agarose gel. Lane a:100 bp DNA ladder; lanes b to e: FIV (pFIV-14), HIV(pILIC), EIAV(p29) and CAEV (pc63-7e5) amplified with the LV1-LV2 set of primers. Note a fragment of 450 bp. Lanes f to i: same order of plasmids amplified with the LV3-DDMY set of primers. Note a fragment of approximately 250 bp.

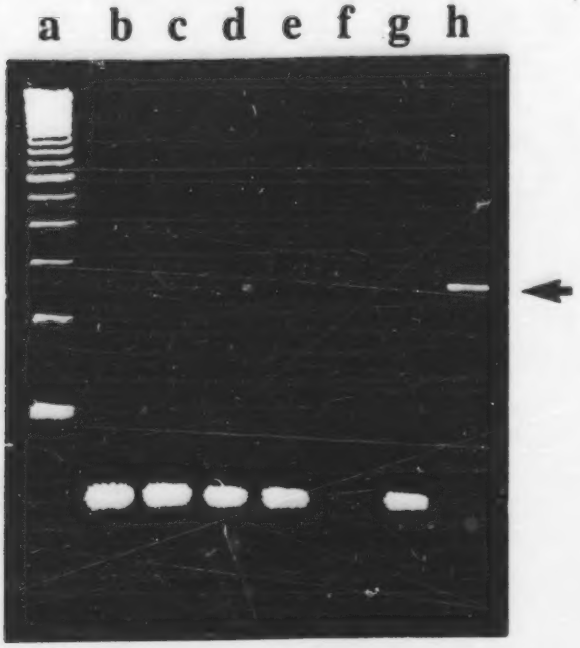

Figure 2. PCR reaction with the LV3-DDMY set of primers on different tissues from affected and normal animals. After a first round of PCR amplification using the LV1-LV2 set of primers, DNA was eluted in the area corresponding to the 450 bp band and 1 ul was used for the second amplification. Lane a: 100 bp DNA ladder; lane b: bone marrow from a normal pig; lane c: bone marrow from an affected pig; lane d: pool of blood leukocytes from 15 affected animals; lane e: pool of blood leukocytes from 5 affected animals from a different herd than lane d; lane f: pool of blood leukocytes from 5 normal animals; lane g: PK15 cells (negative control); lane h: plasmid pILIC (positive control).

Project Activities, Achievements, Papers, Presentations

A manuscript based on these data (essentially the technical report section of this report) has been accepted for publication in the *Canadian Journal of Veterinary Research.* As well these data have been presented or referred to in several intramural and extramural seminars, including the annual meeting of the American College of Veterinary Pathologists. These forums will be an efficient means of transmission of these findings.

Indications for Further Technical or Commerical Development

These data indicate no need at present to develop a screening test for lentiviruses in pigs.

Information of Benefit to Producer's, Government or Industry

These data indicate that a new lentivirus is apparently not involved as a primary pathogen in PMWS. From the standpoint of producers and veterinarians, ruling out the involvement of lentiviruses probably carries a better prognosis for control through vaccination (against circovirus). There currently are no vaccines for any lentiviral disease, due to the difficulty designing vaccines for these rapidly mutating agents (for example HIV, the cause of AIDS).

Personnel

Dr. Ana Bratanich was employed as a post-doctoral fellow during this project, and Carrie Konoby (Technician level II) was employed throughout the duration of the project.

Eqiupment

A PCR machine and multichannel pipetter was purchased for this project and are still in use for investigations into the pathogenesis of PMWS.

Project Developed Materials

None produced or anticipated.

Project Photos

There are photographs of PCR gels which can be made available upon request.

SADF Acknowledgement

The support of SADF has been acknowledged in the CJVR manuscript, as well as in several seminars related to PMWS.

Expense Statement

The statement of expenditures has been forwarded directly to the SADF offices from the University of Saskatchewan Office of Research Services.

Short Communications

Lack of Evidence of Conserved Lentiviral Sequences in Pigs with Post Weaning Multisystemic Wasting Syndrome

Ana Bratanich, Michael Lairmore, Walid Heneine, Carrie Konoby, John Harding, Keith West, Guillermo Vasquez, Gordon Allan, and John Ellis

ABSTRACT

In order to investigate the role of retroviruses in the recently described porcine postweaning multisystemic wasting syndrome (PMWS) serum and leukocytes were screened for reverse transcriptase (RT) activity, and tissues were examined for the presence of conserved lentiviral sequences using degenerate primers in a polymerase chain reaction (PCR). Serum and stimulated leukocytes from the blood and lymph nodes from pigs with PMWS, as well as from control pigs had RT activity that was detected by the sensitive Amp-RT assay. A 257-bp fragment was amplified from DNA from the blood and bone marrow of pigs with PMWS. This fragment was identical in size to conserved lentiviral sequences that were amplified from plasmids containing DNA from several lentiviruses. Cloning and sequencing of the fragment from affected pigs, however, did not reveal homology with the recognized lentiviruses. Together the results of these analyses suggest that the RT activity present in tissues from control and affected pigs is the result of endogenous retrovirus expression, and that a lentivirus is not a primary pathogen in PMWS.

RÉSUMÉ

Dans le but d'étudier le rôle des rétrovirus dans le nouveau syndrome du dépérissement multisystémique post-sevrage porcin (DMPP), du sérum et des leucocytes furent analysés pour détecter la présence d'activité de la transcriptase réverse (TR). De plus, des tissus furent examinés pour vérifier la présence de séquences lentivirales conservées à l'aide de la réaction d'amplification en chaîne par la polymérase (ACP) à l'aide d'amorces dégénérées. Du sérum et des leucocytes stimulés provenant du sang et des ganglions lymphatiques de porcs atteints de DMPP, de même que de porcs témoins, présentaient une activité de la TR. Un fragment de 257 pb fut amplifié à partir du sang et de la moelle osseuse de porc atteint de DMPP. Ce fragment était de dimensions identiques aux séquences lentivirales conservées qui ont été amplifiées à partir de plasmides contenant de l'ADN provenant de plusieurs lentivirus. Le clonage et séquençage du fragment provenant des porcs malades n'ont pas permis de démontrer d'homologie avec les lentivirus connus. Les résultats des analyses suggèrent que l'activité de TR présente dans les tissus d'animaux témoins et malades est le résultat de l'expression d'un rétrovirus endogène, et qu'un lentivirus ne serait pas le pathogène primaire du syndrome DMPP.

(Traduit par le docteur Serge Messier)

Postweaning multisystemic wasting syndrome (PMWS), is a new disease that most often affects 5- to 12-week-old pigs, and has been recognized in swine herds in Canada (1–3), the United States (4), and Europe (4). Clinically, the disease is characterized by progressive weight loss, tachypnea, dyspnea, and jaundice (2). Lesions in affected pigs include lymphadenopathy, lymphocytic to granulomatous interstitial pneumonia, lymphocytic to granulomatous hepatitis, and nephritis (3,4). Previously we have demonstrated a strong association between the lesions of naturally occurring PMWS and the presence of a new porcine circovirus (3,4). Recently we have reproduced the lesions of PMWS in gnotobiotic pigs using chloroform-extracted filtrates of tissue homogenates and cultured viruses derived from affected pigs (5).

Although we have ruled out the primacy of many other known porcine pathogens in the condition (3,4), and reproduced the lesions of PMWS with inocula that are unlikely to contain infectious enveloped viruses, such as retroviruses, many of the clinical and pathologic features of PMWS,

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TABLE I. Detection of reverse transcriptase (RT) activity in the serum of pigs affected with postweaning multisystemic wasting syndrome (PMWS) and clinically normal pigs

Pig #	RT activity	Source	Clinical status
750-1	+	affected herd 1	PMWS
750-2	+	affected herd 1	PMWS
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41	+	affected herd 2	normal
B43	+	affected herd 2	normal
B44	+	affected herd 2	normal
PSWC-1	−	unaffected herd	normal

TABLE II. Detection of reverse transcriptase activity in primary cell cultures

Cells	Days in culture	Source of cells				
		PMWS[a] 1	PMWS 2	PMWS 3	PMWS 4	Control
PBMC[b]	1	ND	ND	ND	−	+
PBMC	3	+	+	+	−	−
PBMC	7	+	+	+	−	+
BLN[c]	1	−	+	−	ND	−
BLN	3	−	+	+	ND	+
BLN	7	+	+	ND	ND	+
PAM[d]	1	−	−	−	−	ND
PAM	3	−	−	−	−	ND
PAM	7	−	−	−	−	ND

[a] pig affected with postweaning multisystemic wasting syndrome
[b] peripheral blood mononuclear leukocytes
[c] bronchial lymph node cells
[d] pulmonary alveolar macrophages

including wasting and lymphoproliferative lesions, are reminiscent of those caused by lentiviruses such as human immunodeficiency virus (HIV) (6), ovine lentivirus (OvLV) (7), caprine encephalitis virus (CAEV) (8), jembrana disease virus (JDV) (9), and feline immunodeficiency virus (FIV) (10). Moreover, since it is possible that several pathogens may act in a synergistic fashion in the genesis of PMWS, a search for retroviral involvement in the condition was justified.

Endogenous porcine retroviruses have been recognized for more than 20 years (11–14). Currently there is renewed interest in porcine retroviruses, due primarily to the use of pig tissues in xenotransplantation in human beings. Recent research has focused on identifying factors that control porcine retroviral expression and examining the potential of these agents as pathogens in transplant recipients. Available data indicate that there are multiple copies of endogenous retroviruses in the DNA of domestic pigs, and a new full length endogenous retroviral sequence has recently been described (12,14). These studies have used miniature and Yucatan pigs, and there is currently little known about endogenous retroviruses in conventional crossbred pigs. To our knowledge, there has been no evidence to date of a porcine lentivirus.

The objectives of this study were twofold. Firstly to screen for retroviruses in pigs with PMWS using a sensitive generic assay to detect reverse transcriptase activity, and, secondly, to examine tissue from affected pigs for lentiviral proviral sequences. Since lentiviruses have yet to be described in pigs, we employed PCR technique with degenerate primers which amplify conserved nucleotide sequences from a wide variety of lentivirus, assuming a new lentivirus would share some homology with them.

Tissues, including blood, liver, lung, kidney, lymph nodes, and bone marrow were collected from debilitated 5- to 12-week-old pigs exhibiting clinical signs (2) and multisystemic lesions (1,3,4) that are characteristic of PMWS. Identical tissues were collected from similarly aged, clinically normal control pigs from a farm which has not had a case of PMWS. In addition, PCV-tested porcine kidney (PK-15) cells (Dr. A. Afshar, Animal Diseases Research Institute, Nepean, Ontario) and Madin-Darby bovine kidney (MDBK; American Type Culture Collection, Rockville, Maryland, USA) cell lines were also used as negative controls.

Blood was collected from the anterior vena cava into acid-citrate-dextrose-containing vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of blood/citrate buffer (50% v/v) mixtures layered on Ficol Hypaque (1.077 specific gravity; Sigma Chemical Company, St. Louis, Missouri, USA), washed twice in phosphate-buffered saline (PBS), and resuspended at 5×10^6 cells/mL in RPMI medium supplemented with HEPES, 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 IU/mL)/streptomycin (100 μg/mL) (supplemented RPMI). Bronchial lymph nodes were aseptically removed and placed in cold RPMI medium in petri dishes on ice. The lymph nodes were manually dissociated, larger fragments were allowed to settle for 5 min, and the cell-rich supernatant was collected. Lymph node cells were resuspended at 5×10^6/mL in the identical supplemented medium that was used for PBMC. A total of 10^7 PBMC or lymph node cells were cultured in 2 mL of supplemented RPMI in wells of 24-well plates and were stimulated with 2.5 mg/mL concanavalin A. Pulmonary alveolar macrophages (PAM) were obtained as previously described (15). The PAM were resuspended in the supplemented RPMI medium at 10^6/mL. PAM were not treated with mitogen. Bone marrow was aspirated from the femur via the trochanteric fossa using a Jamshidi needle (Source Medical, Mississauga, Ontario). Approximately 1 mL of marrow aspirate was suspended in 15 mL of supplemented DMEM with 5% FBS, 1 mg/mL bovine serum albumin, 80 μg/mL transferrin, and 10^{-5} M hydrocortisone.

The Amp-RT assay was performed as previously described (16). Briefly, this assay measures the ability of a sample to produce a DNA copy of a known heterologous RNA template, and the complementary DNA is detected by PCR amplification and Southern blot hybridization. This assay has previously been shown to detect enzyme activity from a wide variety of retroviruses representing all 3 subfamilies of retroviruses from several host species, including ungulates, and is approximately 100 000 times more sensitive that a standard RT assay (16).

DNA and RNA used in PCR reactions to detect lentiviral sequences were extracted from tissue samples and cell lines using Tri Reagent

according to recommendations of the manufacturer (Molecular Research Center, Cincinnati, Ohio, USA).

Two sets of primers, termed LV1: 5′-CCGGATCCDCAPyCCNGSAGGPyTAMAA-3′ (d: A+T+G; Y: C+T; M: A+C), LV2: 5′-GGTCTAGAPyPuPyAPuTTCATAACCCAKCCA-3′ (r: A+G; k: T+G), LV3: 5′-CCGGATCCGAPyPuTPuGGKGAPyGCMTA-3′, and DDMY: 5′-CCGGATCCRTCRTCCATRTA-3′, were used in nested PCR as described (17). The design of the primer sequences were based on conserved reverse transcriptase regions of the polymerase gene of lentiviruses (17). These primers do not amplify sequences from other known exogenous retroviruses (oncoviruses), nor endogenous retroviruses (17). The experimental conditions for nested PCR amplifications using *Taq* polymerase (Promega, Madison, Wisconsin, USA) were identical to those described (17). To assess the integrity of extracted DNA, amplification of a sequence from the beta globin gene in the PCR reactions was assessed by (18). Each reaction was performed in a total volume of 25 μL containing 0.1 to 1 μg of DNA. The PCR products were separated in 2% agarose gels and visualized under UV light after staining with ethidium bromide. A slice of agarose gel was cut in the area corresponding to a size of approximately 450 bp (expected size of the amplification product obtained by the first set of primers, LV1-LV2) and DNA was eluted (Genclean kit, Bio/Can Scientific, Mississauga, Ontario). Subsequently, different amounts of eluted DNA were used to amplify a 257-bp fragment using primers LV3-DDMY. In some experiments, the DNA samples from healthy and affected animals were pooled and used for PCR amplification.

Four different recombinant plasmids containing sequences for the following lentiviruses were used as positive control in PCR reactions: feline immunodeficiency virus (pFIV-14; National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA); caprine arthritis-encephalitis virus (pc63-7e5; Dr. P. Cheevers, Washington State University, Pullman, Washington, USA), equine infectious anemia virus (p29; Dr. W. Maury, University of South Dakota), and human immunodeficiency virus (pILIC; Dr. W. Maury, University of South Dakota).

Two micrograms of extracted RNA and 500 ng of reverse primer (LV2) were used for the synthesis of cDNA in a final volume reaction of 20 mL using standard experimental conditions (19). Subsequently, the synthesized cDNA was used as a source for PCR amplifications as described above. The 257-bp PCR products eluted from agarose gels were cloned into a pCRII vector according to recommendations of the manufacturer (Invitrogen, Carlsbad, California, USA). Plasmid DNA from positive recombinants was extracted using a previously described procedure (20) and the LV3-DDMY primers were used to confirm the presence of an insert. Subsequently, nucleotide sequencing was performed using an automated sequencing system (ABI PRISM, Perkin-Elmer, Foster City, California, USA).

Reverse transcriptase activity was detected in the serum of randomly chosen pigs with typical signs and lesions of PMWS. As well, RT activity was detected in the serum of clinically normal age-matched pigs from herds diagnosed with cases of PMWS, as well as from a closed herd that has never had a known case of PMWS (Table I). Reverse transcriptase activity was also detected in the supernatants of mitogen-stimulated blood mononuclear leukocytes and lymph node cells from a clinically normal pig and pigs with PMWS. Enzymatic activity was not detected in PAM cultures from 4 pigs with PMWS, 3 of which had moderate to severe interstitial pneumonia (Table II).

To test the ability of the degenerate primers to amplify lentivirus sequences, PCR reactions were performed on 4 recombinant positive plasmids. The recombinant plasmids pFIV-14, pc63-7e5, p29, and pILIC used in nested PCR reactions show, as expected, products 450 bp and 257 bp in length with LV1-LV2 and LV3-DDMY primers, respectively (Fig. 1). Subsequently, nested PCR reactions were performed on DNA extracted from liver, spleen, lymph node, lung, blood, and bone marrow from normal and affected animals. A major amplification product of 257 bp was detected in DNA extracted from

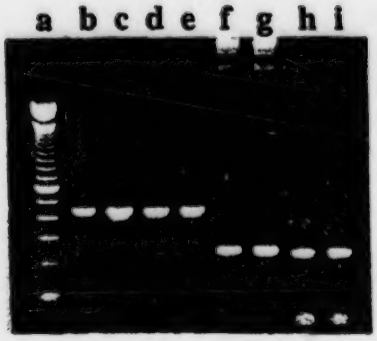

Figure 1. Amplification of different lentiviruses by the LV1-LV2 and LV3-DDMY sets of primers. Ten μL of reaction products were run in a 2% agarose gel. Lane a: 100 bp DNA ladder; lanes b to e: FIV (pFIV-14), HIV(pILIC), EIAV(p29) and CAEV (pc63-7e5) amplified, respectively, with the LV1-LV2 set of primers. Lanes f to i: same order of plasmids amplified with the LV3-DDMY set of primers. Note a fragment of approximately 250 bp.

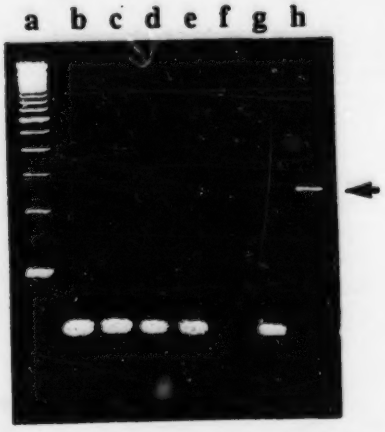

Figure 2. PCR reaction with the LV3-DDMY set of primers on different tissues from affected and normal animals. After a first round of PCR amplification using the LV1-LV2 set of primers, DNA was eluted in the area corresponding to the 450 bp band and 1 μl was used for the second amplification. Lane a: 100 bp DNA ladder; lane b: bone marrow from a normal pig; lane c: bone marrow from an affected pig; lane d: pool of blood leukocytes from 15 affected animals; lane e: pool of blood leukocytes from 5 affected animals from a different herd than lane d; lane f: pool of blood leukocytes from 5 normal animals; lane g: PK-15 cells (negative control); lane h: plasmid pILIC (positive control).

2 separate pools of blood leukocytes from 15 and 5 affected animals from 2 different herds, from the bone marrow of a single affected animal, and in the positive control pILIC. The same amplification product, although

barely detectable, was obtained from DNA extracted from a pool of leukocytes comprising 5 normal animals, and in normal bone marrow sample from a single normal animal, all from a herd that has not had a case of PMWS (Fig. 2). No amplification product was obtained in DNA extracted from liver, lung, lymphoid tissues, spleen, or kidney tissues from affected or normal animals, nor from DNA extracted from PK-15 and MDBK cell lines that were used as negative controls (data not shown). Changes in some reaction parameters, such as the quantity of DNA and annealing temperature, did not alter these results (data not shown). To rule out that the absence of amplification observed in certain tissues was not due the procedure used to extract DNA, a quality control PCR reaction using primers that specifically amplify a region of the beta globin gene was also performed. The presence of a band of the appropriate size was observed in all the samples (data not shown). To determine if a putative porcine lentivirus was actively being expressed in the various tissues of affected animals, RNA samples were also tested by RT-PCR amplification. No amplification product was observed in any samples tested, including blood and bone marrow (data not shown).

Since DNA extracted from affected animals produced a more intense band of the appropriate size than normal tissues, it was hypothesized that along with common cellular sequences, an infectious lentivirus agent could have been amplified in the same reaction. To test this, the 257-bp DNA fragment from blood and bone marrow of affected animals was inserted by T-tail cloning into the pCRII vector. Recombinant clones identified by PCR reactions using the LV3-DDMY set of primers were subsequently sequenced. Comparison of the nucleotide sequences with existing DNA databases did not reveal homologies with lentivirus-like sequences.

Our working hypothesis was that a lentivirus is involved in the pathogenesis of PMWS. To test this hypothesis we first applied a highly sensitive assay of RT activity as a generic test for the presence of retroviruses. The detection of RT activity in serum and supernatants of leukocyte cultures derived from pigs with PMWS was compatible with retroviral infection; however, detection of similar levels of activity in similar samples from clinically normal control pigs suggested that this characteristic enzyme activity was attributable to endogenous retroviral expression, rather than an exogenous retrovirus. Nevertheless, we analyzed DNA extracted from a variety of tissues from affected and normal post weaning pigs using PCR amplification as another sensitive means to determine if this syndrome could be associated with the presence of lentivirus-like sequences. Again, our results did not support the hypothesis.

The primer sequences and conditions used in the PCR reactions were identical to those previously described by Gelman et al (17). These primers were successfully employed to demonstrate the presence of conserved lentiviral polymerase gene sequences from a wide variety of host species in infected cell lines and cell lines containing proviral DNA by use of sets of degenerate primers in a nested PCR reaction (17). We detected the amplification of a product of approximately 250 bp in blood and bone marrow from affected animals, suggesting that lentivirus-like sequences might be associated with PMWS in pigs. However, the sequencing data obtained through the analysis of recombinant clones containing the 257 bp fragment failed to reveal homology with lentivirus sequences. Similar amplification of irrelevant sequences was previously reported using this PCR (17).

There are several explanations for the negative results. The first, and the most obvious explanation is that lentiviruses do not play a role in the pathogenesis of PMWS. The production of lesions typical of PMWS with chloroform extracted filtrates containing porcine circo- and parvoviruses support this possibility (5). Secondly, it is possible that only a few cells containing lentiviral particles or lentiviral provirus were present in the various tissues from affected animals that were examined, and this low level of infection was beyond the limits of detection by PCR amplification. This would be unlike the situation in other species with lentiviral infections, where viremia and lentiviral expression occurs coincident with clinical disease (6–10). However, in this context, it is worth noting that Gelman et al (17) used DNA extracted from established cell lines containing apparently high copy number of lentivirus proviral DNA or lentivirus genes, as well as highly infected cell lines, as templates in PCR reactions. Even with those conditions, they found that these primers did not amplify all lentivirus sequences with the same efficiency and in cases like EIAV and bovine immunodeficiency virus, only 20% of the clones obtained contained lentivirus sequences (17). Thus, to increase the level of sensitivity in detecting expressed lentivirus sequences, we used RNA templates extracted from different tissues in RT-PCR amplification reactions. However, this also failed to reveal the presence of lentivirus-like sequences in tissues of pigs with PMWS. Finally, we used primers that amplify a region of the reverse transcriptase gene that is highly conserved among lentiviruses isolated from different species, assuming there would be similar homology in a putative porcine lentivirus. It is possible, although we believe unlikely, based on lentiviruses described in other species, that the genome of porcine lentivirus is significantly different from that of other known lentiviruses.

In conclusion, we detected inducible RT activity in primary cell cultures derived from crossbred domestic pigs. These data confirm and extend those recently reported in miniature pigs, and indicate an apparently similar level of infection with endogenous retroviruses in genetically diverse domestic pigs (12,13). The fact that RT activity was detected in serum, and at the initiation of PBMC and lymph node cell cultures from some pigs affected with PMWS suggests that the etiologic agent or agents, or the host response to infection, may affect the in vivo expression of endogenous retrovirus. Whether or not such expression of endogenous retrovirus plays a role in the pathogenesis of PMWS is unresolved. Finally, although we cannot completely rule out the possibility that a new porcine lentivirus is involved in the pathogenesis of PMWS, our failure to detect conserved lentiviral

sequences in representative affected pigs with typical clinical signs and lesions of PMWS using these highly sensitive techniques, suggests a lentivirus is not a prevalent or primary pathogen in this emerging infectious disease in swine.

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Porcine circoviruses: a review

Gordon M. Allan, John A. Ellis

Abstract. Porcine circoviruses (PCV) are small nonenveloped DNA viruses containing a unique single-stranded circular genome. Previously, no recognized link was found between PCV infection of pigs and disease, and PCV was considered a nonpathogenic agent. Over the last 5 years, a "novel" PCV, designated PCV2, has been associated with various disease syndromes in pigs, primarily postweaning multisystemic wasting syndrome (PMWS). Pigs with PMWS have a variety of clinical signs, including debility, dyspnea, palpable lymphadenopathy, diarrhea, and pallor or icterus. Lesions associated with the presence of PCV2 in a variety of cell types include lymphohistiocytic to granulomatous interstitial pneumonia, hepatitis, nephritis, myocarditis, enteritis, and pancreatitis. The lesions of PMWS have been reproduced experimentally after inoculation of piglets with PCV2 cell culture isolates, although the full expression of the disease syndrome may require the presence of other agents such as porcine parvovirus or porcine reproductive and respiratory syndrome (PRRS) virus. Recent reports have linked PCV2 to other disorders in pigs, ranging from abortion and reproductive failure to "atypical" PRRS. Available data indicate high seroprevalence of antibodies to PCV2 worldwide. The diagnosis of PCV2-associated disease is based on the direct demonstration of PCV2 antigens or nucleic acid in affected tissues. PCV2 is now regarded as an important emerging pathogen. Although vertical transmission has been documented, the epidemiology of PCV2 infections is poorly understood, as is the role of the immune response in controlling or augmenting disease.

Porcine circovirus (PCV) was first described 25 years ago as a picornavirus-like contaminant of the permanent pig kidney cell culture PK/15 (ATCC-CCL 33).[55] Some 8 years later, the same workers reported PCV as a new, small, nonenveloped, icosahedral mammalian virus containing a circular single-stranded DNA genome.[50] Since this report, PCV has been assigned to the Circoviridae family,[33] and a small body of knowledge on PCV has accumulated in the scientific literature. Recently, the emergence of a new antigenically and genomically distinct PCV in the swine population worldwide, which has been directly associated with a number of clinical conditions, has raised the interest in PCVs. The proposal has been made that this new PCV should be designated PCV2 and the original PCV contaminant of PK/15 cell cultures should be designated PCV1.[8,40] The purpose of this article is to review the current state of knowledge on PCV and provide the swine industry, veterinarians, diagnosticians, and veterinary virologists with a better understanding of PCVs. Within this article, the nomenclature PCV1 and PCV2 will be used as defined above.

Virus isolates

Replication of PCVs in vitro is best achieved by inoculation of semiconfluent monolayers of PCV-free PK/15 cells.[54] A PCV-induced cytopathic effect has not been reported, and viral replication is normally monitored by immunofluorescent or immunoperoxidase staining of infected cell cultures.[3,54] Typically, dense nuclear staining is seen in most infected cells, with occasional large cytoplasmic inclusions in some cells (Fig. 1). Care should be taken with reagents used for immunostaining of PCV-infected preparations because the pattern of staining is virtually identical to that observed with porcine parvovirus (PPV). PCV1 has been shown to replicate in other porcine-derived cell lines,[9,50] and replication in Vero cells has been demonstrated.[3] Prolonged passage of PCV1 in Vero cells has resulted in antigenic changes in the virus, as determined by reactivity against a panel of monoclonal antibodies (MAbs) to PCV1.[3] PCV1 DNA replication has been shown to be dependent on cellular enzymes expressed during S phase of growth, and, in the natural multiplication cycle in vitro, PCV DNA replication starts only if cells have passed mitosis.[54] However, this requirement of mitosis for the onset of multiplication is greatly reduced if cells are infected and subsequently treated with a 300 mM D-glucosamine–HCl solution before they have entered the S phase after growth stimulation.[54] Treatment of inoculated cell cultures with 300 mM D-glucosamine-HCl for 30 minutes is recommended to enhance viral yields, however, care must be taken with this procedure because of the toxic nature of this preparation for cell cultures.

PCV1

PCV1 was first encountered in 1974 and reported as a picornavirus-like contaminant of the continuous pig

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if better understanding and eventual control of the disease syndromes associated with these viruses are to be achieved.

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